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Review article

Conservation of plant genetic resources by cryopreservation

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Abstract

Cryopreservation is a perfect method for long-term conservation of plant genetic resources, using very low temperature (liquid nitrogen, -196°C). This method has been recognized as a practical and efficient tool for the long-term storage of germplasm. Cryopreservation methods may provide the conditions for unlimited conservation of biological materials by reducing metabolic rates. During the cryopreservation all biochemical activities significantly reduced and biological deterioration are stopped. Conservation and subsequent sustainable use of genetic resources are essential to meet the demand for future food security. Several techniques have been developed yet to minimize the damaging effects of desiccation and freezing, ensuring high recovery of plant materials. Cellular division of germplasm is normally repressed after exposure to LN. In addition, metabolic and most physical processes are stopped at this temperature. Thus, plants can be stored for very long time and the problems such as genetic instability and the risk of loose accessions due to contamination or human error during subculture overcome. Techniques like cryopreservation collect and conserve plant genetic resources, especially plants with limited seed storage capability. There is only limited number of plants that cryopreservation techniques are used for their germplasm conservation, mainly because the techniques need to be adapted for each species. Therefore, continued efforts are needed in cryopreservation techniques to develop protocols for a wider range of plants. Formation of ice crystal during cryopreservation is detrimental to cellular structure integrity and causes physical damage to the cells. Air-drying, freeze dehydration, osmotic dehydration, addition of penetrating and non-penetrating cryoprotective substances, and hardening metabolism or combinations of these processes are cryogenic strategies. Nowadays, conservation of plant germplasm has altered from slow cooling to vitrification. However, the availability or developments of simple, reliable and cost-effective strategies and the subsequent regeneration of the plants are basic requirements for germplasm conservation.

Keywords: Cryoprotectant; cryoprotection; encapsulation-dehydration; germplasm collection; *in vitro* culture; plant genetic resources; vitrification; genetic stability.
Abbreviations: ABA-abscises acid; AFLP-amplified fragment length polymorphism; DMSO-dimethylsulfoxide; ED-encapsulation- dehydration; LN-liquid nitrogen; MS-Murashige and Skoog; PCR-polymerase chain reaction; PEG-polyethylene glycol; PVS-plant vitrification solution; RAPD-random amplified polymorphic DNA; RFLP-restriction fragment length polymorphism; SSR-simple sequence repeats.

Introduction

Conservation of plant genetic resources is necessary for food security and agro-biodiversity. Genetic diversity provides options to develop through selection and breeding of new and more productive crops, resistant to biological and environmental stresses (Rao, 2004). For more food, it will be necessary to make better use of a broader range of genetic diversity across the glob. Many plant species are now in danger of becoming extinct (Panis and Lambardi, 2005). More than fifteen million hectares of tropical forests are vanished each year (Rao, 2004). Their preservation is essential for plant breeding programs. Biodiversity provides a source of compounds to the medical, food and crop protection industries (Panis and Lambardi, 2005). Genetically uniform modern varieties are being replaced with highly diverse local cultivars and landraces of traditional agro-ecosystems. Deforestation, urbanization, pollution, habitat destruction, fragmentation and degradation, spread of invasive alien species, climate change, changing life styles, globalization, market economies, over-grazing and changes in land-use pattern are contributing indirectly to the loss of diversity (Pitman and Jorgensen, 2002; Rao, 2004). These reductions are a threat for food security in the long term. Genebanks were established in many countries for conservation of plants (Rao, 2004). Advances in biotechnology, especially in the area of *in vitro* culture techniques and molecular biology provide some important tools for improved conservation and management of plant genetic resources (Ramanatha Rao and Riley, 1994; Withers, 1995). Conservation of plant genetic resources can be carried out either in the natural habitats (*in situ*) or outside (*ex situ*). *Ex situ* conservation is generally used to safeguard populations, in danger of destruction, replacement or deterioration. An approach to *ex situ* conservation includes methods like seed storage in seed banks, field gene banks, botanical gardens, DNA and pollen storage (Rao, 2004). Among these, seed storage is the most convenient method of long-term conservation for plant genetic resources. This involves desiccation of seeds to low moisture contents and storage at low temperatures. Seeds of some species, especially a large number of important tropical and sub-tropical tree species, are recalcitrant or intermediate, i.e. they cannot stand desiccation below a

relatively high critical water content value (10-12% or 20% of fresh weight) (King and Roberts, 1980; Hong et al., 1996) and cold storage without losing viability (Berjak and Pammenter, 1997). Intermediate seeds can be stored by partial drying, although for shorter periods compared to orthodox seeds. Conservation of recalcitrant seeds under humid conditions can be carried out only for short periods, due to germination onset, fungal attack or viability loss (Engelmann and Engels, 2002). The second groups of plants, which are not feasible for seed banking, are vegetatively propagated species (Gonzalez-Benito et al., 2004). They are usually highly heterozygous and, in some cases do not produce seeds, such as banana, sweet potato, sugarcane, cassava, yam, potato and taro (Withers and Engelmann, 1997; Gonzalez-Benito et al., 2004). These species are usually conserved in field gene banks. Field collections loose germplasm (genetic erosion) because of pests, plaque attacks, diseases and adverse weather conditions and their maintenance is labor-intensive and expensive (Panis and Lambardi, 2005). *In vitro* culture is a feasible alternative for genetic conservation of plants where the seed banking is not possible (Henshaw, 1975). *In vitro* culture not only provides a method for clonal propagation and safe exchange of plant material but also used for medium-term germplasm conservation (Withers and Engelmann, 1997; Rao, 2004). Several *in vitro* techniques have been developed for storage of vegetatively propagated and recalcitrant seed producing species (Engelmann and Engels, 2002). In general, they fall under two categories: (1) slow growth procedures, where germplasm accessions are kept as sterile plant tissues or plantlets on nutrient media, which provide short- and medium storage options, and (2) cryopreservation, where plant materials are stored in LN for long-term storage (Engelmann and Engels, 2002). Cryopreservation technique is based on the removal of all freezable water from tissues by physical or osmotic dehydration, followed by ultra-rapid freezing. Cryopreservation includes classical and new techniques. Classical cryopreservation techniques have been developed in the 70-80s. They comprise a cryoprotective treatment followed by slow freezing (Kartha, 1985). The most common cryoprotective substances are dimethylsulfoxide (DMSO), polyethylene glycol (PEG), sucrose, sorbitol and mannitol. These substances have the osmotic actions; however some of them such as DMSO can enter to cells and protect cellular integrity during cryopreservation (Rajasekharan, 2006). Classical cryopreservation methods are mainly used for freezing undifferentiated cultures such as cell suspensions and calluses (Kartha and Englemann, 1994). For freezing of differentiated tissues and organs such as seed, embryonic axes, shoot tips and zygotic and somatic embryos, new techniques include encapsulation-dehydration (ED), vitrification, encapsulation-vitrification, desiccation, pre-growth, pregrowth-desiccation and droplet freezing have been developed (Englemann, 1997). Many of these techniques have been reported for conservation of plants germplasm (Englemann, 2000). For successful cryopreservation, many factors such as source-plant status, starting materials, personnel, culture conditions, pretreatment conditions, cryopreservation methods, cryogenic facilities, regimes and post-thawing are involved (Reinhoud et al., 2000; Reed et al., 2004). Cryopreservation methods include both cryogenic (cryoprotectant and low temperature treatments) and non-cryoprotectant (pre- and post-storage culture) components (Reed et al., 2005). The success of a protocol depends on the tolerance and sensitivity of plant germplasm to the stresses (Reed et al., 2005). Most plant species needs to be conserved at three broad levels; ecosystem level (*in situ*), genotype level (*ex situ*) and gene level (molecular) (Ganeshan, 2006). Cryopreservation and DNA storage may provide long-term storage capabilities. Cryopreservation may be supplemented by DNA storage systems for long-term storage. DNA banks provide novel options for gene banks (Ganeshan, 2006). This technique needs to have further studies to establish as a practical conservation strategy (Shikhamany, 2006).

In vitro culture or slow growth of plant germplasm

Slow growth methods allow plant material to be held for a few years under tissue culture conditions with periodic sub-culturing. In the other word, *in vitro* culture includes some techniques involving the growth under sterile conditions and constant environmental factors of plant germplasm on artificial culture media. Explants are mostly shoot, leaf, flower pieces, immature embryos, hypocotyls fragments or cotyledons (Paunesca, 2009). Generally, younger and more rapidly growing tissues are suitable. The criteria for a proper quality explants are normal, true-to-type donor plant, vigorous and disease free (Fay, 1992). As a rule, fragile tissues including meristems, immature embryos, cotyledons and hypocotyls requires less exposure to sterilizing agents than seeds or lignified organs (Paunesca, 2009). Explants may be obtained from seedlings grown from sterilized seeds. *In vitro* conservation techniques, using slow growth storage, have been developed for a wide range of species, including temperate woody plants, fruit trees, horticultural and numerous tropical species (Shikhamany, 2006). *In vitro* storage based on slow growth techniques has been pointed out as alternative strategies for conservation of genetic resources of plants. In particular, it is useful where the seed banking is not possible, such as vegetatively propagated plants, recalcitrant seed species, and plants with unavailable or non-viable seeds due to damage of grazing or diseases, and large and fleshy seeds. Some species conserved at *in vitro* conditions are *Allium* spp., *Cocos nucifera*, *Theobroma cocoa*, *Vitis*, *Prunus*, *Citrus* spp., *Saccharum*, *Solanum* spp., *Musa* spp., *Colocasia esculentum*, *Manihot* spp., and *Ipomaea batatas* (Henshaw, 1975; Zapartan and Deliu, 1994; Withers, 1995; Ashmore, 1997; Withers and Engelmann, 1997; Engelmann and Engels, 2002; Gonzalez-Benito et al., 2004; Paunesca and Holobiuc, 2005; Sarasan et al., 2006). Some clonal crops are stored in slow-growth medium-term storage as *in vitro* cultures for germplasm conservation (Ashmore, 1997; Benson, 1999). *In vitro* storage of *Zoysia* was successful at 21°C for 2 years (Jarret, 1989). *Lolium multiflorum* Lam. can be kept *in vitro* at 2-4°C with yearly subculture (Dale, 1980). A broad range of *Cynodon in vitro* germplasm remained healthy in storage at 4°C from 4 months to more than 1 year (Aynalem et al., 2002). However, few long-term management options are available for clonal plant germplasm resources (Reed et al., 2005). The development of cryopreservation techniques provides the option for long-term backup of active collections that might otherwise be at risk (Reed et al., 2005). About 37 600 accessions are conserved by slow growth methods in gene banks, worldwide (FAO, 1996). *In vitro* culture provides a method for clonal propagation and short- and medium-term germplasm conservation (Ashmore, 1997). For medium-term conservation, the aim is the reduction of growth, which increases intervals between subcultures. These methods enable extending the subculture periods from 12 months up to 4 years for many species (Ashmore, 1997). In

in vitro conservation, the material can be maintained in a pathogen-tested state and cultures are not subjected to environmental stresses (Withers and Engelmann, 1997). There are several methods, by which slow growth can be maintained. In most cases, a low temperature, often in combination with low light intensity or even darkness, is used to limit growth. Temperature in the range of 0-5°C are employed with cold tolerant species, but for tropical species, which are generally sensitive to cold, temperatures between 15°C and 20°C are used (Withers and Engelmann, 1997). A standard storage treatment for *Pyrus communis* and many other species is 4°C with a 16-h photoperiod for 12 to 18 months (Bell and Reed, 2002). It is also possible to limit growth by altering the culture medium, mainly by reducing the sugar, mannitol or mineral elements concentration, application of abscisic acid (ABA), and reduction of oxygen level, available to cultures. This normally done by covering explants with a layer of liquid medium or mineral oil, or by placing them in controlled atmosphere (Withers and Engelmann, 1997; Engelmann and Engels, 2002). The humidity should be between 40-50% (Paunesca, 2009). Artificial seeds (beads) were first introduced in the 1970s as a novel analogue to the plant seeds, suitable for medium-term storage (Redenbauch et al., 1988). Artificial seeds are produced by encapsulating a plant material in a culture medium containing sodium alginate and then a culture medium containing $CaCl_2$. Plant materials can grow in the proper culture media. Encapsulation is also used for direct protection during dehydration and thawing in cryopreservation (Saiprasad, 2001). Totally, there are three methods for reducing *in vitro* growth rates, including physical (reduced temperature and light conditions), chemical (using growth retardants), and a combination of the two (Engelmann and Engels, 2002). Regeneration and successful propagation of genetically stable seedlings from cultures are prerequisites for any *in vitro* conservation efforts (Ashmore, 1997). Genetically, organized cultures such as shoots are used for slow growth storage, since undifferentiated tissues such as callus are more vulnerable to somaclonal variation (Ashmore, 1997). Advantages of *in vitro* storage include the sterile preservation of materials, no risk of infections by insects or damage through inauspicious weather conditions, less work needed for collections, and the varieties are available all year round (Schäfer-Menuhr, 1996). Disadvantages are that growth retardants change plant morphology and can induce DNA methylation (Harding, 1994), and somaclonal variation (Kumar, 1994). *In vitro* storage based on reduced growth conditions is still labor intensive and there is always the risk of losing accessions due to contamination or human error. Moreover, *in vitro* material of some species is subject to mutations, whose frequency increased during *in vitro* culture.

Cryopreservation

Cryopreservation is a part of biotechnology. Biotechnology plays an important role in international plant conservation programs and in preservation of the world's genetic resources (Bajaj, 1995; Benson, 1999). Advances in biotechnology provide new methods for plant genetic resources and evaluation (Paunesca, 2009). Cryopreservation, developed during the last 25 years, is an important and the most valuable method for long-term conservation of biological materials. The main advantages in cryopreservation are simplicity and the applicability to a wide range of genotypes (Engelmann, 2004). This can be achieved using different procedures, such as pre-growth, desiccation, pregrowth-desiccation, ED, vitrification, encapsulation-vitrification and droplet-freezing (Engelmann, 2004). Cryopreservation involves storage of plant material (such as seed, shoot tip, zygotic and somatic embryos and pollen) at ultra-low temperatures in LN (-196°C) or its vapor phase (-150°C). To avoid the genetic alterations that may occur in long tissue cultures storage, cryopreservation has been developed (Martin et al., 1998). At this temperature, cell division, metabolic, and biochemical activities remain suspended and the material can be stored without changes and deterioration for long time. Walters et al. (2009) proposed that this assumption, based on extrapolations of temperature-reaction kinetic relationships, is not completely supported by accumulating evidence that dried seeds can deteriorate during cryogenic storage. After 30 years of cryogenic storage, seeds of some species exhibited quantitatively lower viability and vigor. In cryopreservation method, subcultures are not required and somaclonal variation is reduced. Advantages of cryopreservation are that germplasm can be kept for theoretically indefinite time with low costs and little space. Besides its use for the conservation of genetic resources, cryopreservation can also be applied for the safe storage of plant tissues with specific characteristics. Different types of plant cell, tissues and organs can be cryopreserved. Cryopreservation is the most suitable long-term storage method for genetic resources of vegetatively maintained crops (Kaczmarczyk et al., 2008). For vegetatively propagated species, the best organs are shoot apices excised from *in vitro* plants. Shoot apices or meristems cultures are suitable because of virus-free plant production, clonal propagation, improving health status, easier recovery and less mutation (Scowcroft, 1984). Seed and field collections have been the only proper for the long-term germplasm conservation of woody species, while a large number of forest angiosperms have recalcitrant seeds with a very limited period of conservability. The species, which are mainly vegetatively propagated, require the conservation of huge number of accessions (Panis and Lambardi, 2005). The storage of this huge number needs large areas of land and high running costs. Preservation of plant germplasm is part of any plant breeding program. The most efficient and economical way of germplasm storage is the form of seeds. However, this kind of storage is not always feasible because 1) some seeds deteriorate due to invasion of pathogens and insects, 2) some plants do not produce seeds and they are propagated vegetatively, 3) some seeds are very heterozygous thus, not proper for maintaining true-to-type genotype, 4) seeds remain viable for a limited time, and 5) clonally propagated crops such as fruit, nut, and many root and tuber vegetables cannot be stored as seed (Chang and Reed, 2001; Bekheet et al., 2007). Cryopreservation offers a good method for conservation of the species, especially woody plant germplasm (Panis and Lambardi, 2005). Cryostorage of seeds in LN was initially developed for the conservation of genetic resources of agriculturally important species (Rajasekharan, 2006). The development of simple cryostorage protocols for orthodox seeds has allowed cryopreservation of a large number of species at low cost, significantly reducing seed deterioration in storage (Stanwood, 1987). Only a few reports are available on the application of cryopreservation on seeds of wild and endangered species and medicinal plants (Rajasekharan, 2006). New cryobiological studies of plant materials has made cryopreservation a realistic tool for long-term storage, for tropical species, which are not intrinsically tolerant to

low temperature and desiccation, has been less extensively investigated (Rajasekharan, 2006). Cryopreservation has been applied to more than 80 plant species (Zhao et al., 2005). Number of species, which can be cryopreserved has rapidly increased over the last several years because of the new techniques and progress of cryopreservation research (Rajasekharan, 2006). The vitrification/one-step freezing and ED methods have been applied to an increasing number of species (Panis and Lambardi, 2005). A new method, named encapsulation- vitrification is noteworthy (Sakai, 2000). The new techniques have produced high levels of post-thaw and minor modifications (Rajasekharan, 2006). In cryopreservation, information recording such as type and size of explants, pretreatment and the correct type and concentration of cryoprotectants, explants water content, cryopreservation method, rate of freezing and thawing, thawing method, recovery medium and incubation conditions is very important (Reed, 2001; González-Benito et al., 2004; Bekheet et al., 2007). All germplasm requires safe storage because even exotic germplasm without obvious economic merit may contain genes or alleles that may be needed as new disease, insect, environmental, or crop production problems arise (Westwood, 1989). It is important to record also the recovery percentage after a short conservation period. A major concern is the genetic stability of conserved material.

Cryopreservation damage, ultra structural changes and cryoprotection

Most plant cells have plenty of water and they are sensitive to freeze. Water content is the single most important factor affecting the ability of germplasm to be stored in LN (Stanwood, 1985). Optimal germplasm water content must be determined. Death or loss of viability will be occurred during the cryopreservation, when the germplasm water content is too much. As the water content decreases, the interaction between water and solutes become stronger, and the system deviates from ideal behavior. On removal of more water, the solution becomes so concentrated that it becomes viscous and has the properties of a glass. At very low water contents, all the remaining water is tightly associated with macromolecular surfaces (bound water) and its mobility is reduced (Vertucci, 1990). Cells must be dehydrated to avoid ice crystal formation (Mazur, 1984). The most damaging event during cryopreservation is the irreversible injury caused by the formation of intracellular ice crystals. Cryopreservation damage of biological material can be caused by physical and biochemical events (Dumet and Benson, 2000). Cryopreservation damages induce based on the physical effects of rice crystal formation and the dynamic effects of freezing rate (Dumet and Benson, 2000). Physical effect accounts for large intracellular ice crystals, which form during rapid cooling and cause mechanical damages. Dynamic effect is the dehydration damage arising from extracellular ice crystal formation. Intracellular ice formation causes damages primarily on membranes (Li et al., 1979; Muldrew et al., 2004). This damage can occur during freezing with ice crystallization or during thawing with recrystallisation of ice. Ultrastructural studies on potato shoot tips showed that the extensive damage was visible after cryopreservation and rewarming (Golmirzaei et al., 2000). These researchers reported cell wall rupture, rupture of epidermis, protoplast outflow and anomalous nucleus shape of surviving and killed explants. Ultrastructural changes during cryopreservation are important to understand and improve this method. The plasma membrane has been considered to be one of the most important determinants for survival at low temperatures (Uemura et al., 2009). The physical damage to the membrane is lethal because this results in the loss of its semi-permeability, imbalance of cytoplasm components, the intrusion of extracellular ice crystals, and many serious injuries in plant cells (Uemura et al., 2009). However, high desiccation also produces damages on cell membrane, due to high concentration of internal solutes and protein denaturation. Thus, plants must increase the cryostability of the plasma membrane to withstand various stresses imposed by freezing and accelerate the recovery process after thawing (Uemura et al., 2009). Uemura et al. (2009) reported that both lipid and protein compositions of the plasma membrane dynamically alter during cold acclimation, which ultimately results in an increase in the cryostability of the plasma membrane. High survival of plant cells after cryopreservation likely requires maintaining the intactness of the plasma membrane. To help plant cells alive, cryoprotectants are often included in the system (Uemura et al., 2009). There are many studies demonstrating that some of the cryoprotectants increase stability of intactness of the plasma membrane through their direct interactions or alterations of water distribution inside/outside cells (Uemura et al., 2009). In their case, plant cells must keep their plasma membrane active and functional for survival. Panta et al. (2009) revealed that freezing resistant genotype of potato have significant higher regeneration rates after cryopreservation and that linoleic acid content is positively correlated with tolerance towards cryopreservation. Also, it was demonstrated that the edition of putrescine to the preculture medium can improve the response of cryopreservation of potato accessions that originally show very low recovery rate (Panta et al., 2009). Zhang et al. (2009) showed the changes of total soluble proteins and calcium in pollen of *Prunus mume* after cryopreservation. One of the best ways to prevent ice crystal formation at LN without damage to membrane and an extreme reduction in cellular water is vitrification, i.e. non-crystalline solidification of water (Panis and Lambardi, 2005). In the other word, vitrification (the production of an amorphous glassy state) circumvents the injurious problems associated with ice formation (Benson, 2004). Two requirements should be performed for vitrification of a solution: 1. enough concentration of the solution, and 2. enough cooling rate of the solution (Panis and Lambardi, 2005). For a solution to be vitrified at high cooling rates, a reduction in water content to at least 20-30% is required. Xu et al. (2009) showed ultrastructural changes during the application of a vitrification protocol of embryogenic cells of *Musa* spp. The results showed that the control cells contained a lot of organelles, a regular nucleolus envelope and intact plasma membrane. After treatment with 25% of PVS2, some changes could be observed, such as smaller vacuoles, more phenol compounds, swollen organelles and appearance of many lipid bodies. Also, multi-vesicular membranous structures with vesicles appeared between the plasma membrane and the cell wall. Moreover, plasmolysis remained limited. Only after dehydration with 100% PVS2, plasmolysis became more severe. Nucleus envelopes in these cells were malformed. Though the cytoplasm and the nucleus became more electron-dense and a lot of heterochromatin appeared, the plasma membrane was still intact. The ultrastructure of cells after freezing, thawing and unloading was similar to that of those cells after dehydration. After 1-2 weeks' of post-thaw recovery, the ultrastructure of surviving cells was similar to that of the

control cells (Xu et al., 2009). A second method for desiccation is using a flow box with a defined air flow, temperature and humidity or alternatively desiccation over various saturated salt solutions (Zamecnik et al., 2009). Following cryogenic strategies result in more concentrated intracellular solutes which most of them associated with cell volume reduction: 1. in air drying or air desiccation, samples are dried by a flow of sterile air under the laminar airflow cabinet. However, there is no control of temperature and air humidity, which both influence strongly the evaporation rate. More reproducible is the air-drying method that uses a closed vial containing a fixed amount of silica gel (Uragami et al., 1990). Studies of Rajasekharan (2006) on conservation of tropical horticultural species showed being better dehydration of germplasm with silica gel than under the laminar air flow. 2. In freeze dehydration, a controlled temperature decrease will also cause cells to dehydrate. During slow cooling, crystallization is initiated in the extracellular spaces. Since, only a proportion of water that contributes to the extracellular solution undergoes transition into ice, the solution becomes more concentrated and hypertonic to the cell. Thus, cellular water will leave the protoplast. Traditional cryopreservation often uses a slow cooling to avoid intracellular ice formation, a common cause of lethal cell damage (Thinh et al., 1999; Lambardi et al., 2000). Equipment is costly, and the method is not effective for low temperature sensitive species (Pennycooke and Towill, 2000). Nowadays, methods to plant germplasm cryopreservation involving direct plunging into LN have been explored, and vitrification procedures proved to be the most promising among them (Lambardi et al., 2000; Tsukazaki et al., 2000). Generally, freezing rates of 0.5 to 2°C/min, and prefreezing temperatures of -30 to -40°C are used. 3. In osmotic dehydration, non-penetrating cryoprotective substances like sugars, sugar alcohols and high molecular weight additives like PEG are applied to the plant tissues. 4. In penetrating and non-penetrating cryoprotective substances, DMSO, glycerol and some amino acids like proline are penetrating substances. DMSO is the best because of its rapid penetration into the cells. DMSO droplet method improved results in potato when applied with alternating temperature precultured (Kryszczuk et al., 2006). Non-penetrating substances are sugars, sugar alcohols and PEG. 5. Hardening is increasing plant ability to environmental stress. Hardening requires a change in metabolism of the cultures triggered by environmental parameters like reduction in temperature and shortening of day length, also, osmotic changes and ABA. Hardening can result in an increase of sugars, proteins, glycerol, proline and glycine betaine, which will act in the increase of osmotic value of the cell solutes. Cold pre-cultures of germplasm before cryopreservation improve results for woody (Niino and Sakai, 1992) and herbal species (Reed et al., 2003; Keller, 2005), which are able to cold-acclimate to low temperature. Some sensitive species which are not able to acclimate to the cold temperatures, cryopreservation could be improved after exposure of germplasm to low temperature (Leunufna and Keller, 2005). Low temperature precultures were successfully used for potato germplasm before cryopreservation using encapsulation-vitrification and droplet-vitrification methods (Hirai and Sakai, 2000; Halmagyi et al., 2005; Kryszczuk et al., 2006; Kaczmarczyk et al., 2008). Studies of Reed (1990) on *Pyrus* showed that regrowth of meristems ranged from 0% to 51% for plants grown at 25°C and 5% to 95% for cold-hardening plants. Cold-hardening significantly improved the recovery rates of all species tested.

Induction of tolerance to dehydration

Dehydration causes some chemical and mechanical damages to the most cells. Sensitivity to dehydration varies among species (Takagi, 2000; Rajasekharan, 2006). The success of a cryopreservation method depends on the tolerance and sensitivity of plant germplasm to the stresses of the cryopreservation method (Reed et al., 2005). Understanding the degree of sensitivity to dehydration is the first step in accomplishing an optimized method (Rajasekharan, 2006). The ability of a germplasm to tolerate dehydration can be achieved by various pretreatments such as sucrose and cold acclimation. Critical phase of the various cryopreservation methods is the pretreatment phase (Rajasekharan, 2006). A number of mechanisms contribute to desiccation tolerance such as: 1. intracellular physical characteristics like reduction of the degree of vacuolation, amount and nature of insoluble reserves accumulated (Farrant et al., 1997), reaction of cytoskeleton (microtubules and microfilaments) (Sargent et al., 1981), and conformation of DNA (Ambika, 2006), 2. intracellular de-differentiation, which minimize surface areas of membranes and cytoskeleton (Ambika, 2006), 3. "switching off" of metabolism (Vertucci and Leopold, 1986), 4. accumulation of protective molecules like some proteins, such as late embryogenic abundant proteins (LEAs) and dehydrines (Kermode, 1990), and sucrose or oligosaccharides (Koster and Leopold, 1988), and 5. the presence and operation of repair mechanisms during rehydration (Ambika, 2006). Synthesis of some proteins associates with the peak in ABA levels (Kermode, 1990; Oliver and Bewley, 1997; Kermode, 1997). Results of the study of Carpentier et al. (2009) suggested that the maintenance of an osmoprotective intracellular sucrose concentration, the enhanced expression of particular genes of the energy-conserving glycolysis and the conservation of the cell wall integrity may be essential to maintain homeostasis and to survive dehydration. These researchers observed a genotype specific expression of certain proteins (isoforms) involved in energy metabolism and ABA- and salt stress- responsive proteins. Carpentier et al. (2009) revealed that twenty eight proteins were correlated to general osmotic stress and fifty nine proteins were exclusively correlated to the sucrose treatment. In choosing cryoprotective treatments or methods, it is important to take into account the origin and physiological status of the germplasm (e.g., temperate or tropical, dormant or active), tolerance to abiotic stresses (e.g., cold and desiccation) as well as operational, technical and practical factors (Reed et al., 2005). The key for successful cryopreservation is shifted from freezing tolerance to dehydration tolerance. Chemical cryoprotective substances like sugars, amino acids, DMSO, glycerol, etc, can induce this tolerance. Sugars, especially sucrose can maintain the liquid crystalline state of the membrane bilayers and stabilize proteins under frozen conditions (Crowe et al., 1984; Kendall et al., 1993). Temperate species that naturally accumulate sugars and protective proteins during seasonal cold accumulation are better able to withstand stresses incurred during cryopreservation as compared to desiccation-sensitive germplasm from tropical species (Reed et al., 2005). Accumulation of sugars increases the stability of membranes under conditions of severe dehydration (Rajasekharan, 2006). Sugars replace the water normally associated with membrane surface, thereby maintaining lipid bilayer (Hoekstra et al., 1991; Crowe et al., 1992). Vitrification of the aqueous phase by sucrose or certain

oligosaccharides leads to glassy state. The presence of glasses, because of their high viscosity, reduce the deleterious effects of deranged metabolism, protecting macromolecules against denaturation and preventing or minimizing liquid crystalline to gel phase transitions in the membrane lipid bilayer (Koster and Leopold, 1988). Progressive increase of the sucrose concentration reduces the toxic effect of high sucrose concentrations, for example the use of media containing 0.3 M, then 0.4 M, then 0.5 M, and finally 0.75 M of sucrose instead of directly placing explants in medium with 0.75 M sucrose. Tolerance to dehydration can also be induced by adaptive metabolism. Cold acclimation in nature often leads to the accumulation of proteins like heat shock proteins, cold regulated proteins, dehydrines, sugars, polyamines and other compounds that can protect cell components during dehydration (Neven et al., 1992; Steponkus et al., 1998). Cold acclimation also alters membrane composition, thereby increasing dehydration tolerance (Sugawara and Steponkus, 1990; Steponkus et al., 1992). Cold-acclimation treatments or treatments that simulate the biochemical base of cold acclimation have been used with great success to increase the cryopreservation survival of temperate and subtropical plant germplasm (Reed and Yu, 1995; Chang and Reed, 1999; Chang et al., 2000). Chang et al. (2000) reported successful of both temperate and subtropical grasses that were cold acclimated for 4 weeks. Exposure to cryoprotectants with lower concentrations than vitrification solution minimizes the damage (Rajasekharan, 2006). Some plant genes are induced by high sugar concentrations (Koch, 1996). Studies of Volk et al. (2009) on *Arabidopsis* shoot tips revealed that cryoprotectant treatments induce gene expression and critical pathways may include those involved in lipid transport and osmoregulation. Alterations in membrane composition, influencing both their flexibility and permeability, are reported (Ramon et al., 2002). Three main categories of seed storage behavior are recognized (Ellis et al., 1990): 1. recalcitrant seeds that cannot withstand dehydration. These are shed at moisture contents more than 50%. Recalcitrant seeds are sensitive to low temperatures and must be kept under high relative humidity conditions. A number of economically important tropical and subtropical crops such as tea, litchi, mango, rubber and forest and horticultural species have recalcitrant seeds (Ambika, 2006). Properties of water in recalcitrant seed tissue are important to know the response to loss of water (Pammenter and Berjack, 2000). 2. Orthodox seeds that survive long-term dry storage. Seeds of most common agricultural and horticultural species such as *Allium cepa*, *Glycine max*, *Cucumis sativus*, *Citrus lemon*, *Capsicum*, *Arachis*, *Amaranthus*, *Melia azedarach*, *Zea mays*, and *Hibiscus esculentus* are tolerant to dehydration and exposure to LN. For these species, critical factor that ensure the survival is seed moisture content (Rajasekharan, 2006). Orthodox seeds can be dried to low moisture contents less than 50% without losing viability. They can be kept successfully for many years at ambient temperatures. 3. Intermediate seeds such as neem, coffee and macrophylla that can withstand dehydration to a certain extent but have reduced longevity (Ellis et al., 1990). These seeds survive drying to moderately low moisture contents (8-10%) but are often injured by low temperatures (Ellis, 1991). Seed moisture content of 4-10% has proved to be better for safe storage of several wild species (Iriondo et al., 1992).

Cryopreservation methods

There are several methods of cryopreservation (Fig. 1). The advantages and disadvantage of each method should be considered. However, other factors such as personnel, available facilities, and type of plant species could influence the selection of the method (Reed, 2001). Cryopreservation methods are different and include the older classic methods based on freeze-induced dehydration of cells as well as newer methods based on vitrification (Engelmann, 2000). New cryopreservation methods include ED, vitrification, encapsulation-vitrification, desiccation, pregrowth, pregrowth-desiccation and droplet freezing (Engelmann, 1997). These methods have been reported for successful use for many cells, tissues and organs of plant species (Engelmann, 2000). The new methods do not need expensive equipment. Cryopreservation is well established for vegetatively propagated species. However, it is much less advanced for recalcitrant seed species in order to some of their characteristics, including their very high sensitivity to desiccation, structural complexity and heterogeneity. Probably, the two first questions to answer are the method to use and the number of replicates required. Various germplasm usually respond differently to the same method, thus researchers must either modify the method for each germplasm or store more explants to compensate for low recovery. Reed (2009) found that standard protocols can be applied to many plants with few if any changes. Screening of groups of plants in a genus shows that many protocols are easily applied to large groups of plants. The protocol to use can be chosen from those developed for similar plants or several standard protocols can be tested (Reed, 2009). Once a protocol is chosen, some critical points can be adjusted to improve the plant response as needed. Each of cryopreservation methods has some basic steps that can be modified to make them effective for many types of plants (Reed, 2009). The overall process for cryopreservation consists of three phase; 1. conditioning of the stock plants, 2. cryogenic conditions including solution application, cooling and warming rates, and 3. recovery processes (Zhao et al., 2005). Cryopreservation methods are as follows (Fig. 1). *Desiccation method (air drying)* Desiccation is the simplest method and consists of hydrating explants and freezing them rapidly by direct immersion in LN. Explants are dried by a flow of sterile air under the laminar airflow cabinet. However, there is no control of temperature and air humidity (Panis et al., 2001). Air drying using a closed vial containing silica gel is more reproducible (Panis et al., 2001). The method is mainly applied to most common agricultural and horticultural species, orthodox seeds, zygotic embryos, embryogenic axes and pollen grain (Uragami et al., 1990; Engelmann, 2004). Some of orthodox seeds are very resistance to drying below 3% moisture content, without any damage and reduction of viability (Uragami et al., 1990; Panis et al., 2001; Engelmann, 2004). When water was removed from the cell, it led to solute effect such as pH changes, increasing electrolyte concentrations, protein denaturation, membrane phase transition and macromolecular interactions and then damage of the cell (Dumet and Benson, 2000). Changrum et al. (1999) suggested that the rate of water loss among different tissues of various species and even among tissues is variable. Thus, drying may not be necessarily beneficial for cryopreservation, if uneven distribution of water results in different freezing responses among cells in the same tissue. Cells with high water content may be predisposed to the volumetric changes of cells would lead to considerable

physical stress within the tissue (Grout, 1995; Reinhoud et al., 2000; Dumet and Benson, 2000).

Slow freezing (classical method)

In this method (also called two-step freezing and slow controlled freezing), tissues are cooled slowly at a controlled rate (usually 0.1- 4°C/min) down to about -40°C followed by rapid immersion of samples in LN. Slow freezing is carried out using a programmable freezing apparatus. Among the various cryopreservation techniques, slow-freezing method seems to be more common (Zhao et al., 2005). This method combines the application of penetrating cryoprotective substances such as DMSO and controlled freeze dehydration, often preceded by cold or sugar hardening or osmotic dehydration. The process of cold acclimation is a multiple trait with complex physical and biochemical change, which alters membrane composition; thereby increasing dehydration tolerance (Sugawara and Steponkus, 1990; Hannah et al., 2005). Cold pre-cultures in tropical and subtropical specie, which are also not able to cold acclimation, have shown improved cryopreservation results (Chang et al., 2000; Leunufna and Keller, 2005). A typical characteristic in cold-acclimated plants is the increased concentration of soluble sugars (Levitt, 1972). Soluble sugars are known to have important function in osmoprotection, cryoprotectant, and metabolization of other protective substances during cryopreservation (Hincha, 1990; Hitmi et al., 1999). In addition, they have hormone-like functions as primary messengers in signal transduction (Rolland et al., 2002). Extracellular ice formation, during freeze-induced dehydration withdraws free liquid water molecules through an osmotic gradient from the cytoplasm to intercellular spaces, where it crystallizes (Benson, 2004). Because of dehydration the cellular concentration of solute rises and becomes too high to nucleate to ice crystals during cooling. This means that it solidifies without crystallization. This status is called the "glassy state". In this state the water molecules are amorphous and lack an organized structure but possess the mechanical and physical properties of a solid (Taylor et al., 2004). Many chemical solutes such as DMSO, sucrose and PVS2 decrease the free water content in cells (Sakai et al., 1990). DMSO and glycerol are cell wall, membrane penetrable, and increase cellular osmolality to avoid ice formation (Benson, 2008). Sucrose can penetrate the cell wall, but not the plasma membrane (Tao and Li, 1986). When cells are frozen, sucrose is concentrated in the cell wall space and protects protoplasts from freeze-induced dehydration. It can form a buffer layer between cell wall and the protoplast to protect the outer surface of the plasma membrane (Tao and Li, 1986). Alteration in essential amino acid was found during cold acclimation. Highest concentrations were found in asparagines, glutamine, glycine and proline during cold acclimation of potato shoot tips (Stewart and Larher, 1980). Most change was in concentration of proline (Stewart and Larher, 1980). It is known that soluble nitrogen content increases in plants grown at lower temperatures (Levitt, 1972). Cooling speed is a main factor of the slow-freezing method of cryopreservation (Benson et al., 1996; Chang and Reed, 2000). In general, the slower cooling rates producing the higher survival rates (Chang and Reed, 2000). Slow freezing, combined with pretreatment by either cold acclimation or ABA has proven to be effective for *Pyrus* germplasm (Bell and Reed, 2002). A comparison of slow freezing and vitrification methods using 28 *Pyrus* genotypes found that regrowth following slow freezing (0.1°C/min) was 61%, while after vitrification was 43% (Luo et al., 1995). Cryoprotectants are added to the freezing mixtures to maintain membrane integrity and increase osmotic potential of the external medium. Classical cryopreservation methods have been successfully applied to undifferentiated culture systems such as cell suspensions and calluses (Kartha and Engelmann, 1994). Fukai (1990) has applied a controlled-rate-freezing method for Chrysanthemum morifolium and other Chrysanthemum species. Tobacco suspension cells were successfully cryopreserved by a vitrification method combined with an encapsulation technique. However, the vitrification method was less effective than simplified slow pre-freezing method (Kobayashi et al., 2006). In potato, survival rate was increased by slow-freezing method as compared with that of the basic cryopreservation method of vitrification alone (Zhao et al., 2005).

Pre-culture and pre-culture/dehydration

Preculture or pregrowth involves preculturing the germplasm on a medium supplemented with cryoprotectants such as sucrose or glucose before exposure to LN. This method is proper for zygotic and somatic embryos of some species (Dumet et al., 1993; Engelmann, 1997a). Also, this simple cryopreservation protocol was successfully applied to highly proliferating meristems of banana (Panis et al., 1996). Meristem cultures are grown for 2 weeks on proliferating medium supplemented with 0.4 M sucrose. Then, surviving meristem clumps are excised, transferred to cryovials and rapidly frozen. Post thaw regeneration rates vary between 0 and 69% depending on the cultivar. Uragami et al. (1990) obtained 63% survival after cooling in LN, in asparagus nodal explants that had previously been precultured for 2 days on 0.7 M sucrose and subsequently desiccated to 20% water content with silica gel. A cryopreservation process using dehydration was performed for seeds of lily [*Lilium ledebourii* (Baker) Bioss.] (Kaviani et al., 2009). Survival after freezing was nil for control seeds and 75% for seeds pretreated with sucrose and dehydration. In *Pyrus*, a 0.75 M sucrose preculture and 4 h dehydration (20% residual water) produced 80% recovery (Scottez et al., 1992). Precultured shoot tips of some species on medium containing high sucrose concentration have been reported to show improved survival of cryopreserved shoot tips (Matsumoto et al., 1995; Takagi et al., 1997; Zhao et al., 2005). However, in some other species, the use of very high sucrose concentration (up to 1 M) was toxic for shoot tip survival (Martinez and Revilla, 1999). Sucrose is an important pregrowth additive for most cryopreservation method, which enhance desiccation tolerance during cryopreservation. Among different types of sugars (fructose, glucose, sorbitol, and sucrose) used as somatic agents in preculture medium, sucrose was the best for the survival of cryopreserved date palm tissue culture (Bekheet et al., 2007). The highest percentage of survival (80%) was observed with 1 M sucrose (Bekheet et al., 2007). Studies on cryopreservation of asparagus (Uragami et al., 1990), carrot (Dereuddre et al., 1991) and date palm (Bagniol and Engelmann, 1992) tissues reveal that survival rates after cryopreservation could be increased by preculturing the tissues on media containing high concentration of sugar (Bekheet et al., 2007). Pretreatment of stock plants with cold acclimation or ABA is very important for cryopreservation of many pear genotypes (Bell and Reed, 2002). Alternating-temperature (22°C for 8-12 h/-1°C for 12-16 h) cold acclimation for 2 to 15 weeks significantly

increase regrowth and recovery remains high for shoots with up to 15 weeks of cold acclimation (Bell and Reed, 2002). Constant temperature acclimation is less effective (Chang and Reed, 2000). Preculture conditions (low temperature and ABA pretreatments) influenced cold hardiness and improved the recovery of cryopreserved *Pyrus cordata* and several other plant shoot tips (Reed, 1993; Vandenbussche and De Proft, 1998). The optimal treatment for recovery was a 3 weeks culture on ABA followed by 2 weeks of low temperature and shoot tips growth increased from zero to 70% (Chang and Reed, 2001). ABA is an important stress hormone produced during cold acclimation (Chang and Reed, 2001). Chen and Gusta (1983) suggested that increased ABA concentration in cells trigger cold acclimation and expression of low temperature-responsive genes. Some plants species need long tolerate temperature treatments, some do not respond to low temperatures, and others do not tolerate low temperature (Chang and Reed, 1997).

Encapsulation-dehydration

In the encapsulation-based techniques, the production of high-quality "synthetic seeds" is required. Although the technique was initially proposed to encapsulate single somatic embryos inside an artificial seed coat (Murashige, 1977), today various other explants such as shoot tips, nodal segments, bulblets, and even callus samples are used to produce synthetic seeds (Lambardi et al., 2006). The ED method requires cryogenic storage in the total absence of ice (Benson et al., 1996). The encapsulation method developed by Redenbauch et al. (1991) is still most widely used to produce synthetic seeds. This method has been developed for apices of numerous species from tropical origin like cassava and sugarcane and of temperate origin like pear, apple, grape and eucalyptus (Dereuddre, 1992; Engelmann, 1997; Sakai, 2004). The method involves the incubation of explants in a Na-alginate solution (1-5%, 3% being the most used) and their subsequent release (immersed in a drop of alginate) into a complexity agent (50-100 mM $CaCl_2$ solution) where bead hardening occurs in 20-30 min. There are two main types of new cryopreservation techniques, ED and vitrification. Combinations of them have also been used. The ED method is based on the artificial seed technology. This method was developed by Fabre and Dereuddre (1990) for *Solanum* and consists of the inclusion of apices (explants) in alginate beads (artificial seeds). Alginate beads can contain mineral salts and organics. The procedure is continued by subsequent culture in a highly concentrated sucrose solution (0.7-1.5 M), followed by physical dehydration or air drying to a moisture content of 20-30% and direct immersion in LN. Culture of explants on sucrose enriched medium (0.3-0.7%) prior to encapsulation, usually improves survival after desiccation and freezing. In the other hand, the presence of a nutritive matrix (the bead) surrounding the explants can promote its regrowth after thawing. ED method is simple, but more handling of alginate beads is required and some species do not tolerate the high sucrose concentrations employed. Physical desiccation is carried out either with silica gel or in the air flow of the laminar flow cabinet (Paulet et al., 1993). Studies of Reed et al. (2005) on *Cynodon* revealed that the ED cryopreservation protocol was most effective, especially when combined with a 1- to 4 week's cold-acclimation period and dehydration to 19 to 23% moisture, before exposure to LN. The moisture content of *Lilium ledebourii* (Baker) Bioss. seeds before exposure to LN were 15-20% (Kaviani et al., 2009). Lily [*Lilium ledebourii* (Baker) Bioss.], Persian lilac (*Melia azedarach* L.), and tea (*Camellia sinensis* L.) were evaluated for long-term storage in LN. Encapsulation within alginate beads was shown to be beneficial in all studied species (Kaviani et al., 2010). Some secondary metabolites were applied as cryoprotectant. Embryonic axes of *Melia azedarach* L. encapsulated into calcium alginate beads with sucrose (0.75 M) and different concentration of salicylic acid were subjected to cryopreservation. Salicylic acid significantly enhanced the percentage of viability of encapsulated embryonic axes (Bernard et al., 2002; Kaviani, 2007). The ED method has been shown to be an effective approach to *Citrus* somatic embryo cryopreservation. 100% survival were obtained after the beads containing somatic embryos had been pretreated on media with high sucrose concentration, dehydrated below 25% moisture content and direct immersed in LN (Duran-Vila, 1995).

Vitrification

For the last ten years, new plant cryopreservation methods have been developed, which are based on vitrification (Uragami et al., 1989). Vitrification (most widely applicable plant cryopreservation method) can be defined as “the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling” (Fahy et al., 1984). Vitrification-based methods involve removal of most or all freezable water by physical or osmotic dehydration of explants, followed by ultra-rapid freezing which results in vitrification of intracellular solutes, i.e. formation of an amorphous glassy structure without occurrence of ice crystals which are detrimental to cellular structure integrity. The vitrification method is easy to perform and often has a high recovery percentage, which makes it widely applicable, particularly to the concentration of plant species sensitive to low temperature (Takagi et al., 1997; Thinh et al., 1999). Vitrification includes of putting explants in a highly concentrated cryoprotective solution, then frozen rapidly. Vitrification involves an increase in cellular viscosity, thus it is important that plants are able to withstand lethal osmotic and evaporative dehydration stresses (Reed et al., 2005). This method has successfully been applied to a broad range of plant materials from various species, including complex organs like embryos and shoot apices (Sakai, 1993; Huang et al., 1995; Takagi, 2000; Vidal et al., 2005; Wang et al., 2005). In the vitrification method, the plant material is exposed to highly concentrated cryoprotectant solutions for variable periods of time (from 15 min up to 2 h), followed by a direct plunge into LN (vitrification/one-step freezing). This results in both intra- and extra-cellular vitrification. Previously, to induce desiccation tolerance, tissues are cultured on medium with high sucrose (0.3 M) or sorbitol (1.4 M) concentration and subsequently transferred to a glycerol-sucrose solution, called loading solution (2 M glycerol + 0.4 M sucrose) (Sakai, 2000). Many reports have shown that osmoprotection with 2 M glycerol and 0.4 M sucrose is effective in enhancing the capacity of cells to tolerate serve dehydration with PVS2 (Hirai and Sakai, 2003; Matsumoto and Sakai, 2003; Kobayashi et al., 2006). A widely used vitrification solution (mixture of penetrating and non-penetrating cryoprotectant substances) is that developed by Sakai et al. (1990) and named plant vitrification solution 2 (PVS2) which consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO in liquid medium with 0.4 M sucrose. Cells are osmotically dehydrated by

PVS2 at a nonfreezing temperature (Sakai et al., 1990). Vitrification requires less handling than ED, but the main problem is the toxicity of the concentrated vitrification solutions. This can be overcome by cold and sugar hardening a loading phase and the application of the vitrification solution at 0°C instead of at room temperature. Vitrification method eliminates the need for controlled slow freezing and permit tissues to be cryopreserved by direct transfer to LN (Kohmura et al., 1992). The treatment of vitrification solution subjects cells to osmotic stress, making it very likely for some constituents to enter into cells and resulting in toxicity (Matsumoto et al., 1994). Thus, careful vitrification solution exposure is critical. Damage to plants because of exposure to vitrification solution may be due to chemical toxicity or osmotic stress (Sakai, 2000). Therefore, delaying permeation of some components of vitrification solution but allowing time for adequate dehydration is critical (Charoensub et al., 1999; Tsukazaki et al., 2000). Direct exposure of germplasm to PVS2 reduces viability and is toxic. The stepwise increase in PVS2 concentration reduces this toxic effect (Kobayashi et al., 2006). Temperate species that accumulate sugars and protective proteins during cold season are better able to withstand as compared to tropical species (Chang et al., 2000; Reed et al., 2005). Improving the tolerance of tropical and warm-temperature plant germplasm to vitrification has been implemented through the use of high-sucrose pretreatments (Benson, 2004; Reed et al., 2004; Sakai, 2004). Substituting sucrose for cold-acclimation treatments may be advantageous for species that are cold sensitive (Niino and Sakai, 1992; Chang and Reed, 2000). The traditional cryogenic technique (slow-cooling) and innovative (vitrification/one-step freezing) methods have all been successfully applied to *Citrus* cryopreservation (Wang and Deng, 2004; De Carlo and Lambardi, 2005). Wang et al. (2005) showed that the recovery percentage of six cultivars' shoot tips of *Carica papaya* L. after vitrification was between 48% and 93%. To determine the potential of vitrification on freezing tolerance of date palm cultures were exposed to a PVS2 for 20-100 min. The maximum rate of survival was obtained with cultures exposed for 80 min at 0°C followed by 40 min at 25°C (Bekheet et al., 2007). Studies of Sarkar and Naik (1998) on potato showed the shoot tips precultured on medium containing 0.3 M sucrose plus 0.2 M mannitol, and loaded with PVS2 for 30 min followed by 15 min incubation in 60% PVS2 and 5 min incubation in 100% PVS2 at 0°C resulted in up to 54% survival after vitrification.

Other methods

Some other cryopreservation methods are the DMSO droplet, droplet-vitrification, two-step vitrification, and encapsulation- vitrification (Fig. 1). Nowadays, DNA storage method also is using. The droplet method can be considered a modification of the previous methods (Schäfer-Menuhr et al., 1997). It was developed for potato germplasm cryopreservation and consists on treating germplasm in drops of a 10% DMSO solution placed on aluminium foil strips, which are rapidly immersed in LN (Schäfer-Menuhr et al., 1994; Mix-Wagner et al., 2003). The term 'droplet' refers to droplets of cryoprotectant on an aluminium foil, into with explants are placed for cooling and rewarming. The original idea of using aluminium foils came from Kartha et al. (1982), who cryopreserved cassava shoot tips on foils in plastic Petri dishes using a two-step cooling method. The foils make it easier to put a large number of germplasm at once quickly into and out of LN. Also, aluminium is a very good heat conductor important for quick cooling and rewarming of samples (Schäfer-Menuhr, 1996). Schäfer-Menuhr et al. (1994) used this idea for a fast cooling method of potato shoot tips. This method has been applied successfully to more than 150 varieties (Schäfer-Menuhr et al., 1996). Droplet freezing was successfully performed with *Chrysanthemum* (Halmagyi et al., 2004). Halmagyi et al. (2004) showed that the droplet method, especially when combined with vitrification has the considerable advantage than for the other methods. In recent years the number of species cryopresereved using the combined droplet vitrification method with rapid cooling and rewarming is increasing (Gonzalez- Arnao et al., 2008). Tokatli and Akdmir (2009) showed that the optimized droplet vitrification protocol improved the mean regeneration rates to more than 30% than ED on Fraser photinia. Totally, droplet vitrification is now one of the methods that receives much attention and is moreover on a large scale applied to germplasm collections of for example banana, potato and garlic (Panis et al., 2009). The method is also now under investigation to be applied in collections from other plant species such as sweet potato, taro and pelargonium (Panis et al., 2009). In some cases, the DMSO droplet method after the alternating temperature preculture is applied. New experiments using alternating temperatures (22/8°C day/night temperature, 8 h photoperiod, 7 days) prior to cryopreservation showed improved regeneration for potato shoot tips (Kaczmarczyk et al., 2008). Total concentrations of soluble sugars (glucose, fructose and sucrose) were higher for all accessions after the alternating temperature preculture, which could be the reason for improved cryopreservation results. Zhao et al. (2005) demonstrated that pretreatment of potato plants at 10°C resulted in improvement of cryopreservation results. Some studies developed a new alternative vitrification solutions, modified either from the original PVS2 vitrification solution by increasing glycerol and sucrose and/or decreasing DMSO and PEG concentration, or from the original PVS3 by decreasing glycerol and sucrose concentration (Kim et al., 2009). The application of these vitrification solutions to some species in a droplet-vitrification method revealed that PVS3 were superior to PVS2 (Kim et al., 2009; González-Arnao et al., 2009). More recently, protocols combining the above techniques have been developed and named encapsulation-vitrification method (Sakai et al., 2000). Firstly, germplasms are encapsulated and then submitted to vitrification, without any further physical desiccation. A modified ED cryopreservation protocol based on the replacement of cold acclimation with high-sucrose pretreatment was assessed for the long-term storage of *Ribes* germplasm (Reed et al., 2005). In many temperate species, incubation of germplasm at low temperature (generally 4°C to 10°C), for periods ranging from days to weeks, increases survival after freezing for both types of methods, classical and new (Reed, 1990; Wu et al., 1999). During cold acclimation cellular changes such as numerous smaller vacuoles, more abundant mitochondria and rough endoplasmic reticulum and accumulation of certain proteins occur. DNA and gene banking are applied for especial genetic stocks and all germplasm (Shikhamany, 2006). Cryopreservation and DNA storage may provide long-term storage capabilities (Ganeshan, 2006). Cryopreservation may be supplemented by DNA storage systems for long-term conservation. DNA storage will be initially limited to gene constructs that code for useful characteristics such as disease or insect resistance

(Ganeshan, 2006).

Conservation of ornamental plants

Some of ornamental plants are in danger of becoming extinct. Efforts are being performed for conservation of them. The conservation of ornamental germplasm can take advantage of innovative techniques, which allow preservation *in vitro* (slow growth storage) or in LN (cryopreservation) of plant material. Slow growth storage refers to the techniques enabling the *in vitro* conservation in aseptic conditions by reducing markedly the frequency of periodic subculturing without affecting the viability and regrowth. Little coordinated effort has been made up to date to conserve and protect the genetic resources of ornamental plants. One exception is the genebank of the "Ornamental Plant Germplasm Center" in USA (Ozudogru et al., 2010). Seed banks are a common way of conserving plant genetic resources. In these, orthodox and sub-orthodox seeds are stored at temperature of either -15°C to -20°C (cold tolerant species) or 0 to -5°C (temperate and tropical species). However, as the seeds do not represent the genetic profile of the mother plant, this approach cannot be used when endangered clonal germplasm is to be preserved, e.g., that of ancient cultivars (Ozudogru et al., 2010). For vegetatively-propagated species, the conservation of clonal germplasm is made in field (clonal collections). However, genotypes preserved only this way run the risks of biotic and abiotic stresses. Today, important complementary approaches to seedbanks and clonal orchards are offered by biotechnology with the possibility to preserve *in vitro* (slow growth storage) or at -196°C (cryopreservation) plant material.

Slow growth storage of ornamental plants

In this method, depending on the species, subculturing can be decreased to once in every several months (sometimes even to 1-2 years) and, because of this, the technique is considered a "medium-term conservation" method. The most widely used approach to slow growth storage of plant material is the coupling of a low temperature (2-5°C for temperate species and 15-25°C for tropical species) with the culture in the dark or low light intensity (Reed, 1992; Lambardi and De Carlo, 2003). Low temperature and light intensity have physiological consequences, such as the reduction of respiration, water loss, wilting, and ethylene production, which allow for safe conservation. In addition to cold storage, *in vitro* conservation can be achieved by modifying the medium compositions, i.e., by 1. reducing the sugar and/or mineral concentration, 2. using growth retardants (e.g., chlorocholin chloride and ABA) or osmotically active compounds (e.g., mannitol), and 3. covering the explants with a layer of liquid medium or mineral oil to reduce the oxygen available to the plants (Withers and Engelmann, 1997). These methods can lead to the appearance of somaclonal variations and thus are not so advisable when true-to-type material is required (Ashmore, 1997). To date, only a limited number of reports, such as *Camellia* spp. (Ballester et al., 1997), *Humulus* spp. (Reed et al., 2003), *Nerium oleander* and *Photinia fraseri* (Ozden-Tokatli et al., 2008), *Splachnum ampullaceum* (Mallón et al., 2007), and *Rosa* (Previati et al., 2008) deal with the *in vitro* conservation of ornamental plants, all based on the cold storage approach. Ornamental plants are generally stored just a few degrees above the freezing (mainly, at 4-5°C) (Ozudogru et al., 2010). Storage in total dark conditions is preferred for a better slowing down of cell metabolism; however, storage under low light intensity showed to be effective for shoot cultures of *Camellia* (Ballester et al., 1997), and *Humulus* spp. (Reed et al., 2003). In general, maximum time of conservation of shoot cultures in cold conditions ranges from some months to 1 year. As for synthetic seeds of ornamentals, they have a shorter time of conservability, ranging from 1.5 to 9 months (Ozudogru et al., 2010). Two exceptions are *Hibiscus moscheutos* (almost 20 months of storage) (West et al., 2006) and *Splachnum ampullaceum* (30 months) (Mallón et al., 2007).

Cryopreservation of ornamental plants

In ornamental species, one-step freezing techniques (such as vitrification, encapsulation-vitrification and ED) are widely preferred than slow cooling (Ozudogru et al., 2010). Examples are *Chrysanthemum grandiflora* (Halmagyi et al., 2004), *Humulus* spp. (Reed et al., 2003), *Acer mono* (Park et al., 2005), *Gentian* spp. (Tanaka et al., 2004), *Dianthus caryophyllus* (Halmagyi and Deliu, 2007), and *Ribes* spp. (Johnson et al., 2007). Protocorm-like bodies of *Oncidium*, precultured with high concentrations of sucrose and glycerol, maintained their cell shape and subcellular components and remained intact after desiccation and freezing, while the cellular structures of protocorm-like bodies, which were not pre-cultured, were damaged (Miao et al., 2005). Halmagyi and Pinker (2006) tested four different sugars or sugar alcohols (i.e., sucrose, glucose, mannitol, and sorbitol) in preculture and observed that the tolerance to freezing of rose shoot tips was highest when sucrose was used. Pretreating explants at 10°C or below (cold hardening) is another approach for inducing freezing tolerance (Ozudogru et al., 2010). For example, nodal segments of *Chrysanthemum* stored at 10°C and low light intensity for 3 weeks, shoot cultures of *Photinia fraseri* stored at 4°C in darkness for 2-3 weeks (Ozden-Tokatli et al., 2008). Also *in vitro*-grown gentian plants stored up to 50 days at 5°C and low light intensity (Tanaka et al., 2004), and embryogenic callus of *Aesculus hippocastamum* stored at 4°C for 5 days in darkness (Lambardi et al., 2005), then plunged in LN. The time of loading explants in PVS2, as well as of dehydrating beads under air flow or on silica gel, should be carefully determined for each species. In ornamental plants, the PVS2 treatment ranges from 5 min (shoot tips of *Chrysanthemum grandiflora*) (Halmagyi et al., 2004) to 3 h (encapsulated shoot tips of *Dianthus caryophyllus*) (Halmagyi and Deliu, 2007). Lynch et al. (1996) reported that 2 h dehydration of encapsulated shoot tips on silica gel was sufficient to induce 25% of germination of *Rosa multiflora* after storage in LN. Encapsulated shoot tips of *Photinia fraseri* needed to be dehydrated 8 h under the sterile air of a laminar flow hood to achieve tolerance to ultra-rapid freezing in LN (Ozden-Tokatli et al., 2008). Warming temperatures ranging from 20°C (Johnson et al., 2007) to 45°C (Reed et al., 2003; Lambardi et al., 2005) have been proposed for ornamental species (Ozudogru et al., 2010). Several orchids were cryopreserved using the ED and vitrification methods (Lurswijidarus and Thammasiri, 2004; Thammasiri and Soamkul, 2007). Some orchid seeds with moisture content lower than 14% can be conserved in LN (Pritchard, 1995; Wang et al., 1998). Halmagyi et al. (2004) reported deep-freezing of shoot tips of *Chrysanthemum morifolium* by different technical methods: controlled- rate-freezing, ED,

ultra-rapid-freezing by the droplet method and vitrification. While vitrification yielded the highest shoot regeneration rates, the droplet method was also successful. Cryopreservation of *Lilium ledebourii* (Baker) Bioss. germplasm by encapsulation-vitrification, and ED methods as well using sucrose and dehydration as pretreatment was performed (Kaviani et al., 2008; Kaviani et al., 2009; Kaviani, 2010; Kaviani et al., 2010). Cryopreservation using sucrose and dehydration showed that survival seeds of lily after freezing were nil for control seeds and 75% for seeds treated with 0.75 M sucrose and dehydration for 1 h in laminar flow (Kaviani et al., 2009). Cryopreservation of lily seeds by ED was revealed that survival rate was nil for control seeds, 22% for seeds treated with sucrose 0.6 M sucrose and dehydration for 1 h in laminar flow and up to 50% for seeds treated with 0.6 M sucrose and dehydration for 1 h and encapsulation (Kaviani et al., 2008). Also, studies on cryopreservation of lily germplasm (seeds, embryonic axes, lateral buds and bulblets) demonstrated about 10% of cryopreserved seeds and embryonic axes pretreated with PVS2, sucrose (0.75M) and encapsulation were able to sprouting, while there was no survival after LN storage of seeds and embryonic axes pretreated with PVS2 and sucrose (0.75 M). None of lateral buds and bulblets pretreated with sucrose (0.75 M) and encapsulation-vitrification was survival after cryopreservation (Kaviani et al., 2010). Our studies showed that the best lily germplasm is seed and the best pretreatments for survival of lily germplasm after cryopreservation are 0.75 M sucrose and dehydration for 1 h (Kaviani et al., 2008; 2009; 2010).

Evaluation of genetic stability and diversity after cryopreservation

The risk of genetic instability has always been a cause of alteration. In theory, metabolic activities at temperatures of LN are reduced to zero, so that after rewarming from cryopreservation, true-to-type plants are expected (Panis et al., 2001). Cryopreserved tissue should be genetically identical to non-treated phenotype and can directly produce normal plants (Dumet and Benson, 2000). A large number of reports showing no evidence of morphological, cytological, biochemical, or molecular alterations in plants from storage at -196°C (Harding, 2004). In the cryopreservation process, some genomic alterations may be induced, thus the determination of genetic integrity is necessary after storage in LN (Ashmore, 1997). The ability to identify genetic variation is important to effective management and use of genetic resources. Genetic instability and somaclonal variations may be caused some differences in genotype and phenotype profiles of cryopreserved plants (Harding, 2004). So, viability and genetic stability are two important factors after cryopreservation (Anand, 2006). Many cryopreservation methods have caused analysis of genetic stability at different levels, but the number of chromosome and its morphology are primary cytogenetic parameters that must remain stable after cryopreservation (Surenciski et al., 2007). Alteration of ploidy level is one of the most frequent genetic variations in *in vitro* systems (Larkin and Scowcroft, 1981). Chromosomal instability is also influenced by the genotype and tissue culture conditions (Surenciski et al., 2007). Methods for assessment of plant genetic diversity (traditionally toward advanced) are summarized as follows: Phenotypic or morphological characters → Biochemical methods (protein and enzyme electrophoresis) → Molecular methods (isozyme markers and DNA-based methods such as RFLP, PCR, RAPD, AFLP, SSR and microsatellite) (Karp et al., 1997; Rao, 2004; Harding, 2004). Traditionally, diversity is assessed by phenotypic characters such as flower color, growth habitat or quantitative agronomic traits like yield potential, stress tolerance, etc (Rao, 2004). The recovered orchid plantlets from cryopreservation by ED technique showed normal growth characteristics (Lurswijid and Thammasiri, 2004). Studies of Marassi et al. (2006) on rice showed 80% of the seedlings developed into normal plants after being transferred to greenhouse conditions. Histological observations showed that the origin of the plants was not modified by cryopreservation process. Studies of Kobayashi et al. (2005) on cryopreservation of tobacco suspension cell cultures revealed that there were no differences in the morphology or growth profiles between cryopreserved cell cultures and the original cell cultures. Shoot tips of *Solanum tuberosum* L. exhibited normal developmental patterns after regeneration from cryopreservation. Cytological studies revealed that their ploidy status was constant and chromosomal abnormalities were not observed (Benson et al., 1996). After phenotypic characters, biochemical methods based on seeds protein and enzyme electrophoresis were introduced (Rao, 2004). Proteins are useful for genetic study because they are the primarily products of structural genes. Even change of single amino acid can be detected in electrophoresis (Anand, 2006). Use of biochemical methods eliminates the environmental influence; however, their usefulness is limited due to their inability to detect low levels of variation (Rao, 2004). Molecular markers have increasingly been used to study genetic diversity from natural populations, plant breeding, identify redundancies in the collections, test accession stability and integrity, and formulate efficient sampling strategies to obtain maximum variation for genetic resources conservation (Rao, 2004). Molecular techniques proved useful in a number of ways to improve the conservation and management of plant genetic resources (Rao, 2004). Molecular markers were used as tools to fingerprint trifoliate orange and sunflower germplasm accessions (Fang et al., 1997; Hongtrakul et al., 1997). Variation within species has been studied to show geographic or ecological patterns of distribution of diversity in many crops such as banana (Pillay et al., 2001), sorghum (Nkongolo and Nsapato, 2003), tea (Balasaravanan et al., 2003) and sweet potato (Gichuki et al., 2003). Molecular markers are being used to resolve problems of taxonomy and phylogenetic relationships to determine of genomic homologies in devising proper breeding strategies for appropriate conservation and gene transfer (Ramanata Rao and Riley, 1994; Rossetto et al., 2002; Rao, 2004). Molecular markers can be used for characterization of germplasm, varietal identification and clonal fidelity testing, assessment of genetic diversity, validation of genetic identification and marker-assisted selection (Anand, 2006). Enzymes encoded by different alleles at one or more gene loci are known as allozymes and isozymes, respectively. Isozyme markers were found to be more specific than total protein patterns (Anand, 2006). A total of about 90 isozyme systems have been used for plant assessment (Paunesca, 2009). The advantages of this technique are that is relatively simple and less expensive (Anand, 2006). The main limitations of isozyme analysis are the reduction of number of analyzed markers and the phenotypical, developmental and seasonal dependence of the markers (Paunesca, 2009). Examples of the use of molecular markers to study genetic diversity have been

reported in plants such as flax (van Treuren et al., 2001), barley (Lund et al., 2003), wheat (Cao et al., 1998), sorghum (Dean et al., 1999), and grapevine (Cervera et al., 1998). DNA-based markers have been applied after enzyme markers (Anand, 2006). DNA- based markers are derived from the initial template DNA and provide the best measure of genetic variation (Anand, 2006). DNA- based techniques have potential to identify polymorphisms represented by differences in DNA sequences in nuclear and organelles and is not modified by environmental exposure. Also, the analysis of DNA can be carried out at any time during plant development and it may cover the entire genome (Paunesca, 2009). These techniques analyze the variation at the DNA level that includes all environmental influences too (Rao, 2004). The analysis can be performed at any growth stage using any plant part and it requires only small amounts of material (Rao, 2004). DNA-based analysis was applied to study the genetic stability of plant tissue culture due to its high sensitivity and accuracy in detecting every single base change (Shuji et al. 1992). Molecular methods, e.g., RFLP (restriction fragment length polymorphism), PCR (polymerase chain reaction), RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), SSR (simple sequence repeats) or microsatellite were used for detecting DNA sequence variation are based on the use of restriction enzymes that recognize and cut specific short sequences of DNA. RFLP analysis of mitochondrial DNA was used for assessing polygenetic relationship in *Solanum* spp. (Isshiki et al., 2003). PCR and RFLP are applied to endangered species (Chase and Fay, 1997). Microsatellites have been deployed to establish the genetic stability of long-term maintained germplasm (Paunesca, 2009). Microsatellites include low copy regions of plant genomes (Morgante et al., 2002). Microsatellite profiles in propagules of some potato varieties were identical to those of the parental plants and their progeny (Harding and Benson, 2001). Microsatellites were applied to distinguish different cultivars of grapevine (Thomas et al., 1994), and to compare landraces and develop unique DNA profiles of soybean genotypes (Rongwen et al., 1995). Microsatellites analysis demonstrated high level of polymorphism within and among *Lycopersicon*, which was correlated with cross-pollinating (Alvarez et al., 2001). Tang and Knapp (2003) performed phylogenetic analysis on cultivated and wild germplasm accessions of *Helianthus* using microsatellite loci, which revealed the possibility of multiple domestication origin in *Helianthus*. As a complementary method to molecular markers, flow cytometry is used to detect the possible changes in ploidy levels and DNA content (Fukai et al., 2002). In potato shoot tips using the DMSO droplet method, genetic stability was confirmed using morphological parameters, flow cytometry and RFLP analysis (Schäfer-Menuhr et al., 1997). Storage of *Solanum tuberosum* L. in LN for up to ten years was found to have no adverse effect (somaclonal variation) on the regeneration rates (Mix-Wagner et al., 2002; Keller et al., 2006). DNA markers are extremely useful for testing clonal fidelity (Anand, 2006), a key position in breeding strategies, genetic engineering, genome mapping, DNA fingerprinting, genetic variation, cultivar identification, characterization of genotypes in plant germplasm collections, identification of genetic contamination and quantification of genetic drifts/shifts and taxonomic studies (Ananthanarayanan, 2006). Newer molecular techniques are permitting precise and versatile analyses of genetic variation. But, it is difficult to compare the different techniques and determine which one is best and for what purpose because each method has its advantages and disadvantages (Rao, 2004). The appropriateness of individual marker systems varies depending on the objective of study and the properties of the species (Karp et al., 1997). Surenciski et al. (2007) analyzed *Cyrtopodium hatschbachii* cytogenetically and found that the cryopreserved encapsulated seeds were stable at chromosome and phenotypic level. However, limited condensation of the chromatin during the first stages of their development was observed. In *Swietenia macrophylla*, changes in chromatin conformation after recovery from LN were observed, possibly due to changes in methylation status of the DNA (Harding and Millam, 2000). Studies confirmed the stability of the ribosomal RNA genes and the nuclear-chloroplast DNA in potato plants regenerated from cryopreserved shoot apices (Harding, 1991; Harding and Benson, 2000). Xu et al. (2002) determined the variation in chloroplast DNA simple sequence repeats in wild and cultivated soybean accessions, and indicated a higher genetic diversity in the wild species. In apples, the methylation status of DNA was altered after storage in LN and the changes were accompanied by an increase in the capacity of cryopreserved shoot tips for rooting (Hao et al., 2001). DNA methylation patterns are stable and inherited, resulting in the phenomenon of DNA imprinting (Shemer et al., 1996). Methylation changes in genomic DNA after cryopreservation were found in chrysanthemum shoots (Martin and Gonzalez-Benito, 2006), potato (Harding, 1997), and almond leaves (Channuntapipat et al., 2003). DNA methylation can play a role in somaclonal variation (Kaeppler et al., 2000), but Channuntapipat et al. (2003) and Harding (2004) suggest that these changes may not be induced by cryopreservation but are the results of the whole process of *in vitro* culture and regeneration. Studies of Scocchi et al. (2004) on cryopreservation of apical meristem-tips of *Melia azedarach* L. using encapsulation/dehydration showed that this cryopreservation treatment preserved genetic stability, when it was evaluated using the electrophoresis pattern of nine isozymes and RAPD bands. The regenerated plants appeared morphologically similar to the control ones (Scocchi et al., 2004). RAPD technique has been used to study the genetic stability of cryopreserved tissue cultures of date palm (Bekheet et al., 2007). According to RADP analysis, plantlets derived from cryopreserved cultures were identical to that derived from non-treated cultures and both were similar with the field growth plants (Bekheet et al., 2007). Codominant isozymic markers as well as dominant RAPD markers are valuable tools to look for differences to ensure genetic stability (De Loose and Gheysen, 1995). Genetic variation within and between natural populations of *Digitalis obscura* was quantified using RAPDs and the results were used for optimizing sampling strategies for conservation of genetic resources of the species (Nebauer et al., 1999). RAPD analysis in *Brassica oleracea* demonstrated that 14 phenotypical uniform accessions could be reduced to 4 groups with minimal loss of genetic variation (Phippen et al., 1997). RAPDs were used to identify dwarf off-types arising from micropropagation of banana cultivars and changes in genetic diversity following regeneration of potato and wheat accessions (Damasco et al., 1996; Del Rio et al., 1997; Börner et al., 2000). AFLP marker analysis revealed genetic diversity within and between some accessions such as sweet potato and *Coffea arabica* (Fajardo et al., 2002; Steiger et al., 2002). AFLPs were applied to validate taxonomic classification in wild potato species (McGregor et al., 2002). New molecular

techniques detect variation at specific gene loci, permitting precise and versatile analyses of genetic variation (Sicard et al., 1999; Rao, 2004). PCR is too sensitive and pathogen specific for explants health testing, which are applied for elimination of systemic diseases for safe exchange of germplasm (Rao, 2004).

Cryopreservation of organs and tissues of plants

All parts of plants may be conserved by cryopreservation. Suspension or callus cultures, dormant buds, apical meristem, embryonic axes, seeds, somatic embryos and pollen are now stored in LN (Bell and Reed, 2002). Suspension cells and calluses are often cryopreserved using the classical slow-cooling method (0.5°C/min up to -40°C). The main goal of cryopreserving these tissues is the conservation of specific features that can be lost during *in vitro* conditions (Panis and Lambardi, 2005). Cryopreservation of cell suspensions and calluses is reported for some species (Panis et al., 2004). Many successful methods are based on the slow-cooling method consisting in the pretreatment of embryogenic callus of *Citrus* with cryoprotectants, mainly DMSO and sucrose before being slowly cooled to -40°C and then immersed in LN (De Carlo and Lambardi, 2005). The PVS2 was developed working with embryogenic cells of *Citrus* spp. (Sakai et al., 1990). Tobacco suspension cells were successfully cryopreserved by a vitrification method combined with an encapsulation technique. However, the vitrification method was less effective for cryopreservation than a simplified slow freezing method (Kobayashi et al., 2006). Pollen grains are cryopreserved for breeding programs, distributing and exchanging germplasm among locations, as well as for studies in some sciences such as physiology, biotechnology and *in vitro* fertilization (Towill and Waters, 2000). In *Citrus*, the survival of ovules to cryopreservation has been shown to be very erratic, but the pollen has been successfully stored in LN (De Carlo and Lambardi, 2005). Storage of orthodox seeds in LN is an alternative to the traditional storage at -20°C. For seed cryopreservation, dehydration/one-step freezing methods have been successfully applied to some species (Pence, 1995). Embryo axes and intact seeds have been shown to be excellent explants for *Citrus* cryopreservation (De Carlo and Lambardi, 2005). Meristematic tissues are the most common explants for cryopreservation of vegetatively-propagated species, such as fruit trees. Somaclonal variation is lower in organized tissues like meristem than that of non-organized tissues like callus and cell suspensions. ED and encapsulation-vitrification methods are mainly used for meristematic tissues (De Carlo and Lambardi, 2005; Panis and Lambardi, 2005). Shoot tips or apical meristem are widely used for cryopreservation of many species (Lambardi et al., 2001). The explants above described are mainly applied for herbaceous species. Three categories are mainly used for woody plant cryopreservation; 1. shoot tips, 2. seeds or the isolated embryo axes, and 3. embryogenic callus. Different methods have been used for the cryopreservation of embryogenic calluses, shoot tips, ovules and pollen, embryo axes, and seeds from a wide range of *Citrus* species and cultivars (Pérez, 2000).

Recovery of cryopreserved germplasm

Long-term storage can take place in LN or in the vapor phase. For the recovery of germplasm after cryostorage, rapid rewarming is usually required to avoid recrystallisation (Towill, 1991). Vials containing the germplasm are usually immersed in a water bath at 35-40°C. When germplasm are not included in vials, for example in the droplet method, rewarming usually takes place in liquid medium at room temperature (Mix-Wagner et al., 2003). In many species, recovery of apices cryopreserved with the new methods is direct, without callus formation. By contrast with the classic methods, the structural integrity of most cells is well preserved (Engelmann, 1997a). Some studies have shown the importance of the proper post-thawing culture conditions to enhance organized growth. In many cases, selection of a suitable growth medium for germplasm recovery is necessary. Adjustment of growth regulator concentration (Withers et al., 1988) or even medium salt formulation (Pennycooke and Towill, 2001; Decruse and Seeni, 2002) could be required for the normal development of frozen germplasm. In some germplasm conservation centers, 20% recovery is considered enough for long-term preservation (Golmirzaie and Panta, 2000). Other authors consider that survival should be higher than 40% (Reed, 2001). It is important that those percentages be reproducible.

Conclusion

Biotechnology has created significant contributions to improved conservation and use of plant genetic resources. The rapid progress made in *in vitro* culture technology, cryopreservation and molecular markers has helped in improving the plant germplasm conservation and offer a valuable alternative to plant diversity studies, and management of genetic resources. Cryopreservation has proven to be an efficient long-term conservation method for genetic resources. Nowadays, vitrification method is a standardized method, although more studies should be performed. Adjustments of the methods to the genebank would be necessary to exploit all the advantages of cryopreservation. The two most important factors that need to be optimized are the preparation phase of tissues towards dehydration (especially by sugar and cold treatments) and the length of explants treatment with the vitrification solution. Researches should move toward standardizing and simplifying the methods.

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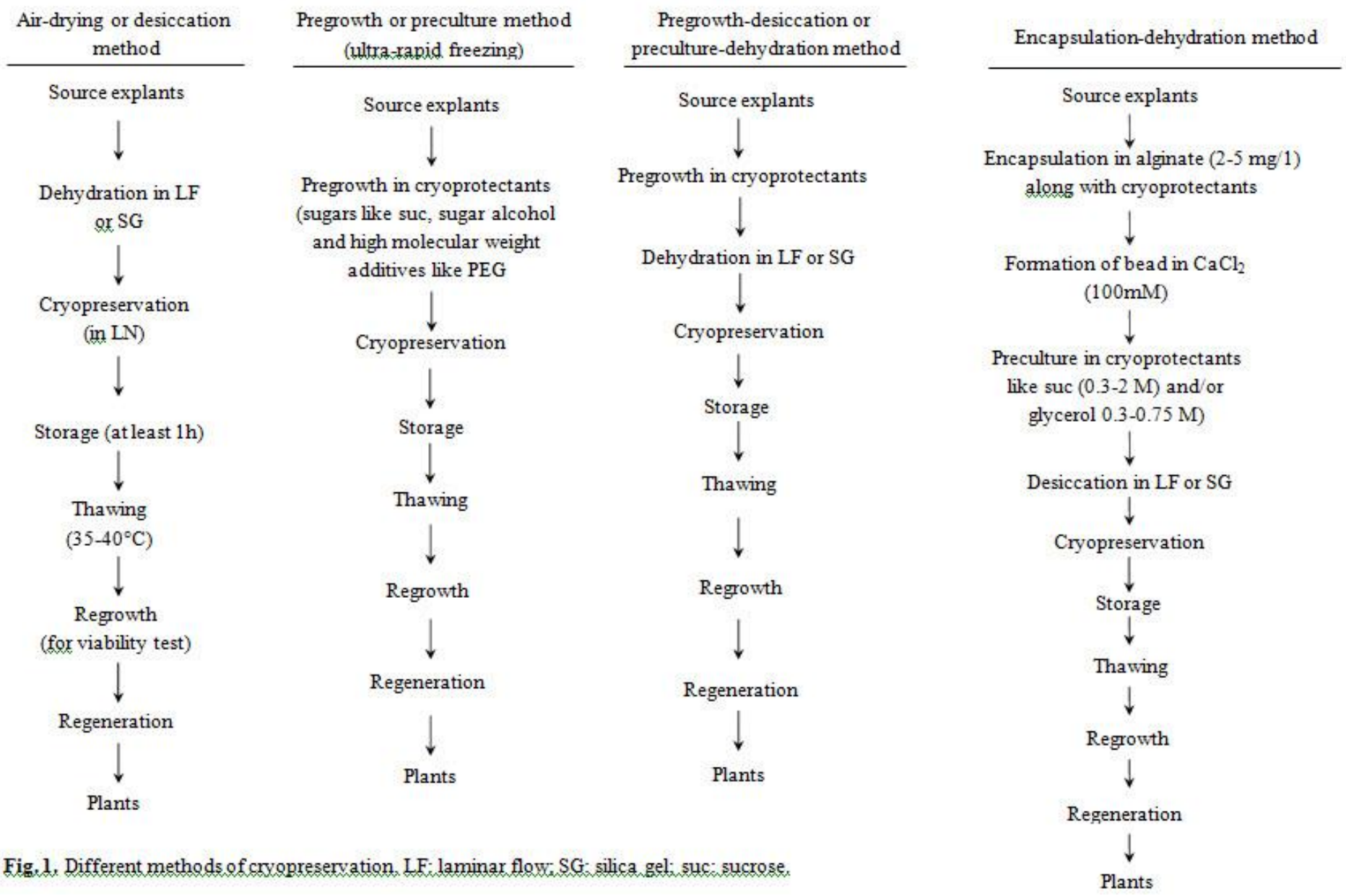

Fig. 1. Different methods of cryopreservation. LF: laminar flow; SG: silica gel; suc: sucrose.

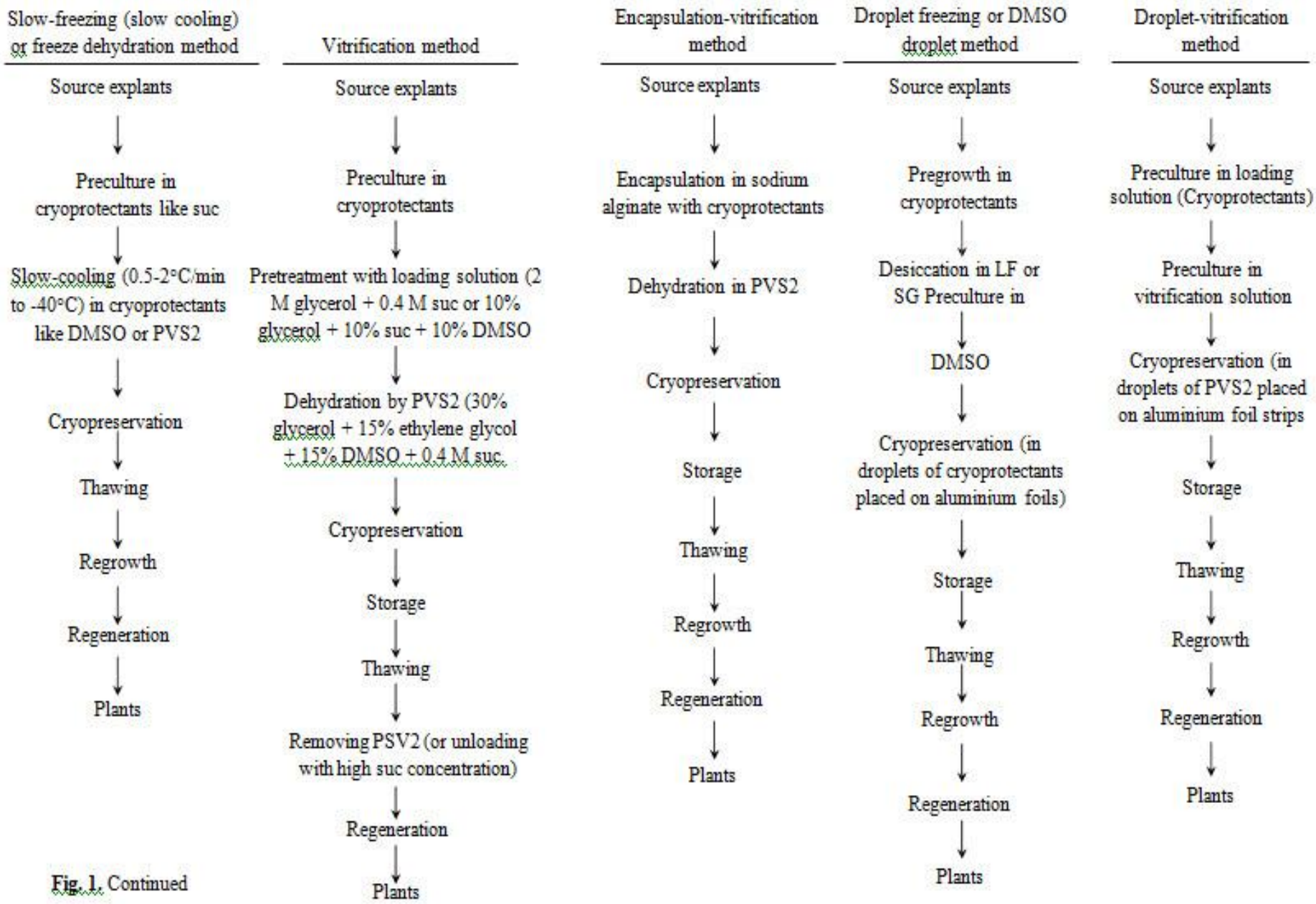

Fig. 1. Continued